

# Anionic Phospholipids and the Alb3 Translocase Activate Signal Recognition Particle-Receptor Interaction during Light-harvesting Chlorophyll a/b-binding Protein Targeting\*

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The universally conserved signal recognition particle (SRP) co-translationally delivers newly synthesized membrane and secretory proteins to the target cellular membrane. The only exception is found in the chloroplast of green plants, where the chloroplast SRP (cpSRP) post-translationally targets light-harvesting chlorophyll a/b-binding proteins (LHCP) to the thylakoid membrane. The mechanism and regulation of this post-translational mode of targeting by cpSRP remain unclear. Using biochemical and biophysical methods, here we show that anionic phospholipids activate the cpSRP receptor cpFtsY to promote rapid and stable cpSRP54-cpFtsY complex assembly. Furthermore, the stromal domain of the Alb3 translocase binds with high affinity to and regulates GTP hydrolysis in the cpSRP54-cpFtsY complex, suggesting that cpFtsY is primarily responsible for initial recruitment of the targeting complex to Alb3. These results suggest a new model for the sequential recruitment, remodeling, and unloading of the targeting complex at membrane translocase sites in the post-translational cpSRP pathway.

Localization of proteins to their correct cellular destinations is essential for proper cellular function. The universally conserved signal recognition particle (SRP)<sup>2</sup> is responsible for co-translationally targeting newly synthesized membrane and secretory proteins to the eukaryotic endoplasmic reticulum or the bacterial plasma membrane (1–3). The conserved functional core of SRP consists of a 54-kDa SRP54 protein and an SRP RNA. SRP54 contains an N-terminal N-domain and a GTP-binding G-domain that together form a structural and functional unit termed the NG-domain. SRP54 also contains a methionine-rich M-domain, which binds to signal sequences

on nascent proteins as they emerge from the ribosome (4–6). Upon cargo recognition, SRP bound to the translating ribosome is brought to the membrane via the GTP-dependent interaction of its NG-domain with a highly homologous NG-domain in the SRP receptor (SR) (7, 8). At the membrane, the SRP-SR complex unloads its cargo to the SecYEG or Sec61p translocase, which mediates the integration or translocation of the nascent protein across the membrane (9, 10). In addition, GTP hydrolysis is activated in the SRP-SR complex to drive their disassembly, allowing SRP and SR to enter the next round of targeting (11–13).

Nevertheless, a large portion of membrane proteins cannot use the co-translational SRP pathway and instead must use post-translational modes of targeting (14, 15). An example is found in the chloroplast of green plants, where a chloroplast SRP (cpSRP) pathway post-translationally targets the light-harvesting chlorophyll a/b-binding family of proteins (LHCPs) to the thylakoid membrane (16, 17). LHCPs are nuclearly encoded and initially synthesized in the cytosol. Upon import into the chloroplast stroma, fully synthesized LHCPs are recognized by and forms a soluble “transit complex” with cpSRP. The cpSRP pathway preserves the chloroplast homologues of SRP54 and SR (cpSRP54 and cpFtsY, respectively), whose GTP-dependent interaction mediates the delivery of LHCPs to the thylakoid membrane (18, 19). At the membrane, LHCPs are unloaded onto the Alb3 translocase, which mediates the insertion of LHCPs into the thylakoid membrane (20–24). LHCPs are the most abundant membrane proteins on earth and comprise over 30% of the proteins in the thylakoid membrane. Their sheer abundance and crucial role in energy generation of green plants demand a highly efficient and robust targeting machinery and make cpSRP a major protein-targeting pathway in nature.

Besides the universally conserved SRP54 and SR GTPases, cpSRP provides a notable exception to the classic cytosolic SRP pathway. The otherwise universally conserved SRP RNA is no longer present; in our previous work and our accompanying paper (51), we described how the M-domain of cpSRP54 replaces the SRP RNA to enable efficient interaction between the cpSRP54 and cpFtsY GTPases (25–29). In addition, the transition to a post-translational mode of targeting necessitated the evolution of a novel SRP subunit, cpSRP43, which binds with high affinity and specificity to LHCPs and provides an effective chaperone that prevents these multipass integral membrane proteins from aggregation (30, 31). cpSRP43 con-

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<sup>2</sup> The abbreviations used are: SRP, signal recognition particle; SR, SRP receptor; cp, chloroplast; LHCP, light-harvesting chlorophyll a/b binding protein; DACM, N-(7-dimethylamino-4-methylcoumarin-3-yl)-maleimide; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; GppNHp, guanosine 5'-[β,γ-imido]triphosphate; PG, phosphoglycerol; RNC, ribosome-nascent chain complex; CTD, C-terminal domain; SQDG, sulfoquinovosyl diacylglycerol; DGDG, digalactosyldiacylglycerol.

tains a substrate binding domain that recognizes LHCPs and two chromodomains, CD2 and CD3 (32, 33). CD2 binds the C-terminal tail of the cpSRP54 M-domain to form the cpSRP, and CD3 provides an interaction site with the Alb3 translocase (see below) (34, 35).

Studies of the bacterial SRP showed that to achieve efficient and accurate protein localization, the SRP and SR GTPases must be actively regulated by their spatial and temporal cues. Kinetic analyses of these GTPases from bacteria and chloroplast showed that, in the absence of effector molecules, SRP and SR undergo GTPase cycles that are not conducive to efficient protein targeting. Complex assembly between the SRP and SR GTPases in both systems is extremely slow, on the order of  $10^2$ – $10^3 \text{ M}^{-1} \text{ s}^{-1}$  (this study and Refs. 27, 28, 36), and is insufficient to support the demands of protein targeting *in vivo*. Once a stable SRP-SR GTPase complex is formed, GTP hydrolysis occurs at a rate constant of 30–50  $\text{min}^{-1}$  (27, 36), giving the targeting complex a lifetime of 1–2  $\text{s}^{-1}$  before its disassembly. If recruitment of the translocase and unloading of cargo protein onto the translocase were not completed before GTP hydrolysis, abortive reactions would ensue. With bacterial SRP, we have shown that the ribosome-nascent chain complex (RNC) extensively regulates the SRP-FtsY GTPase cycle to overcome these barriers. RNCs bearing SRP-dependent substrate proteins accelerate SRP-FtsY complex assembly  $10^2$ – $10^3$ -fold, thus ensuring rapid cargo delivery to the membrane (37). RNC also delays GTPase activation in the SRP-FtsY complex to minimize abortive reactions (38). These cargo-induced allosteric regulations are critical for the efficiency and fidelity of the co-translational SRP pathway. This raises the following question. In the absence of RNC, what are the biological cues that regulate the interaction, activity, and timing of the chloroplast SRP and SR GTPases during LHCP targeting?

Previous work showed that bacterial FtsY preferentially binds anionic phospholipids, such as phosphoglycerol (PG) and cardiolipin, using an amphiphilic lipid-binding helix at the very N terminus of its N-domain (39, 40). Interaction of PG and cardiolipin with this motif stimulates complex assembly between bacterial SRP and SR by pre-organizing SR into a more active conformation (41). This lipid-binding motif is conserved in cpFtsY and is necessary for targeting LHCP to the thylakoid membrane (42). Although the composition of the thylakoid membrane is different from the bacterial plasma membrane, it also contains ~25% of anionic phospholipids such as PG and sulfoquinovosyl diacylglycerol (SQDG). Total soybean extract liposomes were observed to enhance the basal GTPase activity of cpFtsY by 2-fold (42). Nevertheless, these previous experiments were carried out under conditions that do not detect the interaction between the cpSRP54 and cpFtsY GTPases. The roles of the target membrane in the cpSRP-cpFtsY GTPase cycle remain elusive.

The mechanism by which the targeting complex is recruited to the Alb3 translocase on the thylakoid membrane is also incompletely understood. Alb3 belongs to the YidC/Oxa/Alb3 family of membrane protein insertases required for the proper biogenesis of multiple membrane proteins (22, 43). Alb3 contains five transmembrane helices and a long unstructured C-terminal stromal domain (Alb3-CTD). Full-length Alb3

expresses poorly and is prone to proteolysis (44); nevertheless, a direct interaction between conserved basic motifs on Alb3-CTD and CD3 of cpSRP43 has been demonstrated (20, 35, 44–46). This suggests that Alb3-CTD can contact interaction partners in the absence of its transmembrane domains. Nevertheless, this interaction is fairly weak, with a dissociation constant of 11–17  $\mu\text{M}$ , and it is further weakened by the binding of cpSRP54 to cpSRP43 (35, 47). This and the predominantly stromal localization of cpSRP43 (42) raise questions as to whether the cpSRP43-Alb3 interaction is primarily responsible for recruiting the targeting complex to the Alb3 translocase. Whether and how Alb3 also interacts with and regulates the cpSRP54 or cpFtsY GTPases also remain unclear.

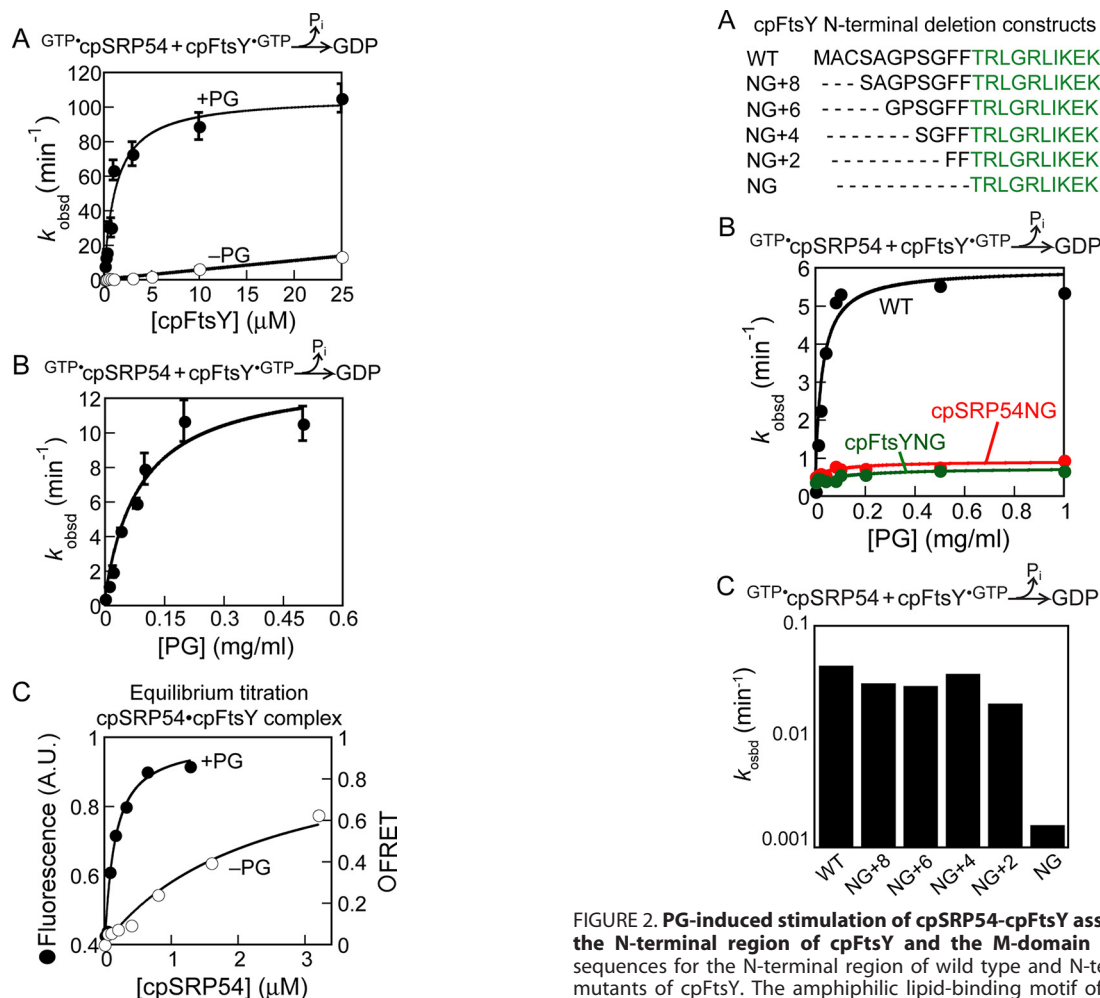
In this work, we address these questions and test the role of phospholipids and Alb3 in regulating the cpSRP54/cpFtsY GTPase cycle. Using biochemical, biophysical, and enzymatic assays, we showed that anionic phospholipids activate cpFtsY for efficient interaction with cpSRP54 and stabilize the cpSRP54-cpFtsY complex. Alb3-CTD binds with submicromolar affinity to the cpSRP54-cpFtsY complex and regulates GTP hydrolysis in this complex. These results demonstrate extensive spatial regulation of the SRP and SR GTPases in the cpSRP system and suggest a new model for the recruitment of the targeting complex to the membrane translocase during LHCP targeting.

## Results

**Anionic Phospholipids Stimulate cpSRP54-cpFtsY Complex Assembly**—To test whether cpFtsY is activated by anionic phospholipids to better interact with cpSRP, we measured the stimulated GTPase reaction between cpSRP54 and cpFtsY. In this well established assay, the reaction is rate-limited by complex formation between cpSRP54 and cpFtsY at sub-saturating cpFtsY concentrations (27). Hence, the value of  $k_{\text{cat}}/K_m$  in this reaction reflects  $k_{\text{on}}$ , the rate constant for cpSRP54-cpFtsY complex assembly (27). Interestingly, the value of  $k_{\text{cat}}/K_m$  is increased 150-fold in the presence of PG liposomes (Fig. 1A). This suggests that, analogous to the bacterial SRP system, complex assembly between the chloroplast SRP and SR GTPases is strongly enhanced by anionic phospholipids.

To test how strongly FtsY interacts with PG, we titrated PG liposomes in the stimulated GTPase reaction (Fig. 1B). Reactions were carried out at sub-saturating FtsY concentrations so that the observed rate constant ( $k_{\text{obsd}}$ ) is rate-limited by cpSRP54-cpFtsY complex assembly. The PG concentration required to reach half-maximal stimulation is 0.09 mg/ml for cpFtsY (Fig. 1B). In comparison, 0.5–1 mg/ml PG was required to attain half-maximal stimulation in the analogous reaction with *Escherichia coli* SRP and FtsY (41). This suggests that cpFtsY binds anionic phospholipids more tightly than its bacterial homolog.

To test whether anionic phospholipids also enhance the stability of the cpSRP54-cpFtsY complex, we carried out equilibrium titrations for formation of the cpSRP54-cpFtsY complex in the absence and presence of PG. Complex formation in the absence of PG was monitored by Förster resonance energy transfer (FRET) between donor *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM) labeled at cpFtsY



**FIGURE 1. Phospholipids stimulate cpSRP54-cpFtsY complex formation.** *A*, representative data for the stimulated GTP hydrolysis reaction between cpSRP54 and cpFtsY, measured in the presence (●) and absence (○) of PG liposomes as described under “Experimental Procedures.” Reactions contained 0.1  $\mu\text{M}$  cpSRP54 and the indicated amounts of cpFtsY, 100  $\mu\text{M}$  GTP, and 0.1 mg/ml plant PG liposomes where applicable. The data were fit to Equation 1 and gave  $k_{\text{cat}}/K_m$  values of  $9 \times 10^3$  and  $1.25 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  in the absence and presence of PG, respectively, and a  $k_{\text{cat}}$  value of 100  $\text{min}^{-1}$  for the reaction in the presence of PG. The  $k_{\text{cat}}$  value for the reaction in the absence of PG could not be determined, and a low limit of 10  $\text{min}^{-1}$  ( $k_{\text{obsd}}$  at the highest [cpFtsY] tested) is reported. Error bars indicate S.E. *B*, stimulated GTP hydrolysis reaction was measured as a function of PG concentration. The data were fit to Equation 2 and yielded a  $K_{1/2}$  value of 0.09 mg/ml for PG liposomes. Error bars indicate S.E. *C*, equilibrium titrations for cpSRP54-cpFtsY complex formation in the presence (●) and absence (○) of PG, measured using fluorescence assays as described under “Experimental Procedures.” Titrations in the presence of PG used 20 nM DACM-labeled cpFtsY, indicated concentrations of unlabeled cpSRP54, and 2 mM GTP. The data were fit to Equation 5 and gave a  $K_d$  value of 130 nM. Titrations in the absence of PG were measured by FRET using 50 nM DACM-labeled cpFtsY, indicated concentrations of BODIPY-labeled cpSRP54, and 2 mM GTP. The data were fit to Equation 6 assuming a FRET end point of 1, which gave an upper limit for  $K_d$  of 2.34  $\mu\text{M}$ . A.U., arbitrary units.

(Cys-321) and acceptor (BODIPY-fluorescein-*N*-(2-aminoethyl)-maleimide) labeled at cpSRP54(Cys-234) (Fig. 1C, open circles) (29). Complex formation in the presence of PG was monitored by the fluorescence enhancement of DACM labeled at cpFtsY(Cys-321) (Fig. 1C, closed circles). In the presence of GTP, the equilibrium dissociation constant ( $K_d$ ) for cpSRP54-cpFtsY binding was 130 nM in the presence of PG, ~20-fold lower than that in the absence of PG (Fig. 1C). Together, these

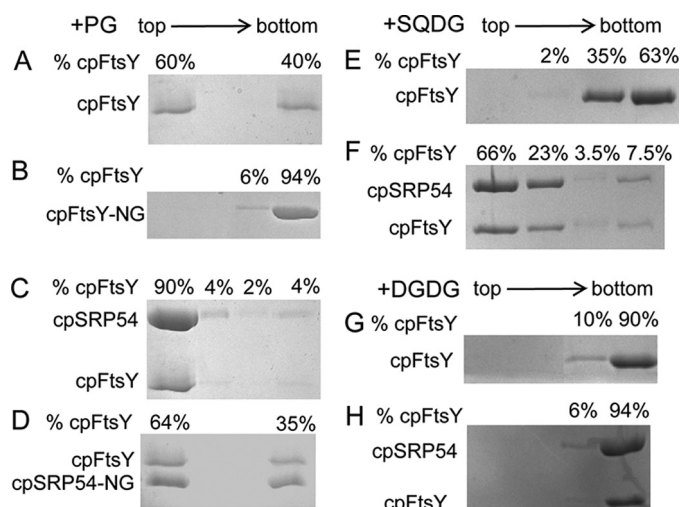
**FIGURE 2. PG-induced stimulation of cpSRP54-cpFtsY assembly requires the N-terminal region of cpFtsY and the M-domain of cpSRP54.** *A*, sequences for the N-terminal region of wild type and N-terminal deletion mutants of cpFtsY. The amphiphilic lipid-binding motif of cpFtsY is highlighted in green. *B*, representative data for the stimulated GTP hydrolysis reaction of wild type cpSRP54 with cpFtsY (black), cpSRP54-NG with cpFtsY (red), and cpSRP54 with cpFtsY-NG (green). The reactions used 0.1  $\mu\text{M}$  cpSRP54 or cpSRP54-NG, 0.3  $\mu\text{M}$  cpFtsY or cpFtsY-NG, and indicated amounts of PG. The data were fit to Equation 2. *C*, apparent rate constants for cpSRP54-cpFtsY complex assembly for wild type and N-terminal deletion mutants of cpFtsY. Stimulated GTPase reactions were measured using 0.1 mg/ml PG, 0.1  $\mu\text{M}$  cpSRP54, 0.3  $\mu\text{M}$  cpFtsY, and 100  $\mu\text{M}$  GTP.

results demonstrate that anionic phospholipids strongly accelerate and stabilize the formation of the cpSRP54-cpFtsY complex.

**cpSRP54 M-domain and N-terminal Region of cpFtsY Are Required for Lipid-mediated Stimulation**—In both bacterial and chloroplast FtsYs, an important lipid-binding motif has been mapped to an amphiphilic helix at the N terminus of the NG-domain (Fig. 2A, green residues (40–42)). Importantly, one phenylalanine in *E. coli* FtsY and two phenylalanines in cpFtsY preceding this motif play critical roles in inducing helix formation in this motif and are hence required for membrane binding of these receptors (40, 42, 48). Consistent with these results, we found that truncation of the N-terminal residues of cpFtsY (to yield cpFtsY-NG) abolished the PG-induced stimulation of GTPase assembly (Fig. 2, A and B, green). To further understand the role of the N-terminal residues in regulating the cpSRP54-cpFtsY interaction, we sequentially deleted pairs of residues N-terminal to the NG-domain (Fig. 2A). Most of the deletion mutants exhibited reaction rates similar to full-length cpFtsY in



## Activation of SRP-Receptor Interaction during LHCP Targeting



**FIGURE 3. cpSRP54-cpFtsY complex binds more tightly to anionic phospholipids than free cpFtsY.** Lipid flotation analysis was carried out as described under "Experimental Procedures" in the presence of PG (A–D), SQDG (E and F), and DGDG (G and H) lipids. With PG, the following samples were analyzed: free cpFtsY (A); free cpFtsY-NG (B); the cpSRP54-cpFtsY complex formed in 1 mM GppNHp (C); and the cpSRP54-NG-cpFtsY complex formed in 1 mM GppNHp (D). With SQDG and DGDG, free cpFtsY (E and G) and the cpSRP54-cpFtsY complex formed in 1 mM GppNHp (F and H) were analyzed. The values above each panel represent quantification of the % cpFtsY in each fraction.

the presence of PG (Fig. 2C). Only when the two phenylalanine residues immediately preceding the NG-domain were deleted did we observe a significant reduction in reaction rate (Fig. 2C). Thus, cpFtsY(NG+2) is the minimal construct required for lipid-induced stimulation of cpSRP54-cpFtsY assembly. These mutational results closely mirror those observed in the binding of cpFtsY to thylakoid membrane or liposomes (42), indicating that the PG-induced activation of the cpSRP54-cpFtsY complex assembly is mediated by this lipid interaction motif of cpFtsY.

As described in the accompanying paper (51), the cpSRP54 M-domain provides a platform to coordinate rapid and stable complex assembly between cpSRP54 and cpFtsY, thus replacing the otherwise conserved SRP RNA (28). Interestingly, deletion of the cpSRP54 M-domain abolished the stimulatory effect of PG on complex assembly (Fig. 2B, red). Thus, both the M-domain of cpSRP54 and the lipid-binding helix of cpFtsY are required for PG-induced stimulation of complex assembly between cpSRP54 and cpFtsY.

**Complex Formation with cpSRP54 Enhances Lipid Binding of cpFtsY**—If anionic phospholipids enhance the stability of the cpSRP54-cpFtsY complex, then reciprocally, complex formation with cpSRP54 should enhance the lipid binding of cpFtsY. To test this prediction, we semi-quantitatively measured the binding of cpFtsY to PG liposomes using density gradient lipid flotation (40, 41). Interestingly, only 60% of free cpFtsY floated with liposomes to the top of the gradient (Fig. 3A). Consistent with previous work and the results in Fig. 2, cpFtsY-NG did not float with PG liposomes (Fig. 3B). In contrast, when a stable cpFtsY-cpSRP54 complex is formed in the presence of the non-hydrolyzable GTP analog, GppNHp, over 90% of the complex floated with lipids to the top of the gradient (Fig. 3C). Consistent with the results in Fig. 2, the cpSRP54-NG-cpFtsY complex lacking the cpSRP54 M-domain showed much less enhance-

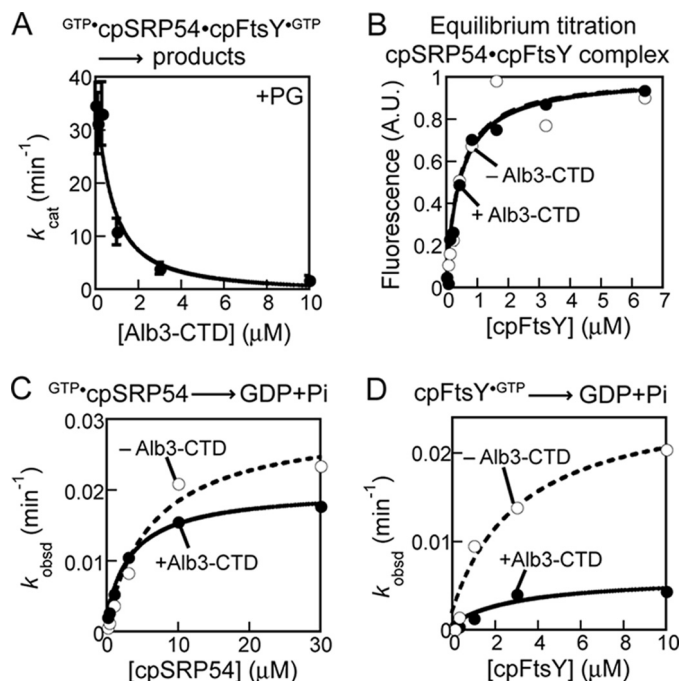
ment in lipid binding (Fig. 3D). These results provide independent evidence that the interaction of cpFtsY with phospholipid membranes and with cpSRP54 enhance one another; further, the M-domain of cpSRP54 plays an important role in this cooperativity.

In addition, we tested the effect of another anionic phospholipid, SQDG, as well as a neutral lipid, digalactosyldiacylglycerol (DGDG), that are found in the thylakoid membrane of chloroplasts. Analogous to observations with PG, the cpFtsY-cpSRP54 complex exhibited enhanced binding to SQDG compared with free cpFtsY (Fig. 3, E and F), although cpFtsY by itself binds less strongly to SQDG than to PG (Fig. 3, A versus E). Neither free cpFtsY nor the cpSRP54-cpFtsY complex floated with the neutral lipid DGDG (Fig. 3, G and H). These results strongly suggest that the membrane interactions of cpFtsY and the cpSRP54-cpFtsY complex are specific to anionic phospholipids.

**Stromal Domain of Alb3 Translocase Regulates GTP Hydrolysis**—Alb3-CTD directly binds the cpSRP43 subunit of cpSRP (20, 44, 45). To better understand the role of Alb3-CTD, we tested whether it can regulate the GTPase cycle of cpSRP54 and cpFtsY. To this end, we carried out the stimulated GTP hydrolysis reaction of cpSRP54 with cpFtsY at saturating cpFtsY and GTP concentrations, such that GTP hydrolysis from the cpSRP54-cpFtsY complex ( $k_{cat}$ ) was monitored. In the presence of PG liposomes, we found that Alb3-CTD strongly inhibited the GTP hydrolysis reaction from this complex, reducing the value of  $k_{cat}$  from 35 to 1.0 min<sup>-1</sup> (Fig. 4A). The apparent inhibition constant ( $K_{i,app}$ ) was 0.47  $\mu$ M (Fig. 4A), suggesting that Alb3-CTD binds strongly to the GTP-cpSRP54-cpFtsY<sup>GTP</sup> complex.

To test whether Alb3 also affects assembly of the GTP-cpSRP54-cpFtsY<sup>GTP</sup> complex, we performed equilibrium titrations for this complex in the absence and presence of Alb3-CTD. Complex formation was measured by using the environmentally sensitive increase in the fluorescence of acrylodan labeled-cpSRP54(Cys-234) upon binding cpFtsY, as described earlier (29). The binding affinities of cpSRP54 for cpFtsY were the same, within error, with and without Alb3-CTD present (Fig. 4B). In contrast, Alb3-CTD did not affect the basal GTPase activity of free cpSRP54 (Fig. 4C) and only reduced the basal GTPase reaction of cpFtsY by 4-fold in the presence of liposomes (Fig. 4D). The rate constants for the basal GTPase reactions of free cpSRP54 and cpFtsY were over 100-fold slower and thus cannot significantly contribute to the stimulated GTPase reaction of the cpSRP54-cpFtsY complex (cf. Fig. 4, C and D, versus A). Thus, Alb3-CTD inhibits the activated GTP hydrolysis reaction after a stable cpSRP54-cpFtsY complex is formed.

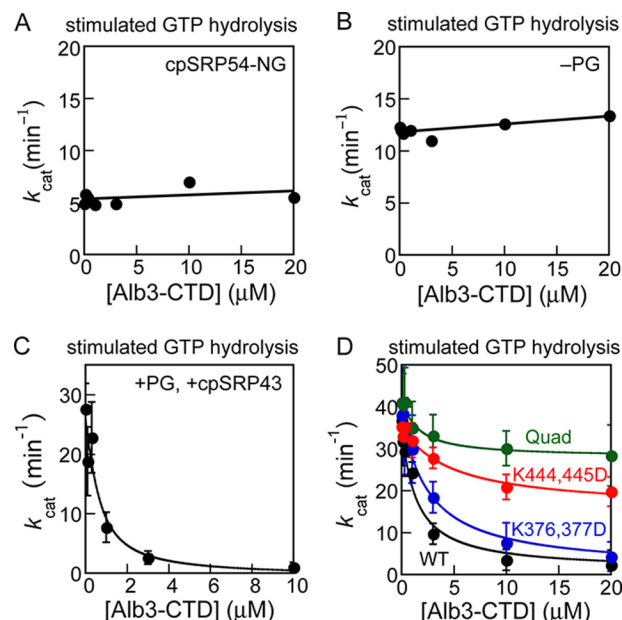
**Molecular Requirements for the Regulatory Effect of Alb3**—To understand the mechanism by which Alb3-CTD delays GTP hydrolysis in the cpSRP54-cpFtsY complex, we first tested the molecular requirements for this effect. To ask whether the cpSRP54 M-domain was required for the Alb3-mediated delay of GTP hydrolysis, we tested the effect of Alb3-CTD on GTP hydrolysis from the cpSRP54-NG-cpFtsY complex (which lacks the cpSRP54 M-domain). No Alb3-CTD-induced changes in GTP hydrolysis rates were observed with cpSRP54-NG (Fig. 5A). Analogously, the effect of Alb3-CTD on GTP hydrolysis of the cpSRP54-cpFtsY complex was abolished in the absence of



**FIGURE 4. Alb3-CTD regulates GTP hydrolysis in the cpSRP54-cpFtsY complex.** A, representative data showing the effect of Alb3-CTD on the GTPase rate constant from the cpSRP54-cpFtsY complex. Reactions contained 0.1  $\mu\text{M}$  cpSRP54, 0.5  $\mu\text{M}$  cpFtsY, 0.1 mg/ml PG liposomes, indicated concentrations of Alb3-CTD, and 100  $\mu\text{M}$  GTP. The data were fit to Equation 4, which gave an inhibition constant of 0.47  $\mu\text{M}$  for Alb3-CTD binding to the cpSRP54-cpFtsY complex, and a  $k_{\text{cat}}$  value of 1  $\text{min}^{-1}$  for the Alb3-bound GTPase complex. B, equilibrium titrations for cpSRP54-cpFtsY complex formation in the presence (closed) or absence (open) of Alb3-CTD. Titrations used 0.1  $\mu\text{M}$  acrylodan-labeled cpSRP54(Cys-234) and indicated concentrations of unlabeled cpFtsY, with or without 7.5  $\mu\text{M}$  Alb3-CTD present. The data were fit to Equation 5 (except that the term [cpFtsY] in the denominator is replaced by [cpSRP54]) and gave  $K_d$  values of 0.340 and 0.368  $\mu\text{M}$  in the presence and absence of Alb3-CTD, respectively. C and D, representative data for the basal GTPase reactions of cpSRP54 (C) and cpFtsY (D) in the presence (closed) and absence (open) of 5  $\mu\text{M}$  Alb3-CTD. Reactions contained indicated concentrations of cpSRP54 or cpFtsY and 0.2 mg/ml plant PG liposomes. The lines were fits of the data to Equation 3. A.U., arbitrary units.

PG liposomes (Fig. 5B). In contrast, the same inhibitory effect of Alb3-CTD on GTPase activation was observed with and without cpSRP43 present (Figs. 4A and 5C). Together, these results demonstrate that the regulatory effect of Alb3 is specific and only occurs in a cpSRP54-cpFtsY GTPase complex formed in the presence of anionic phospholipids and in which the cpSRP54 M-domain is properly positioned near the FtsY GTPase site, but is independent of cpSRP43.

We further asked whether specific interaction motifs in Alb3-CTD mediate its interaction with and regulation of the GTPases. Although Alb3-CTD is predicted to be largely unstructured, previous analyses identified several conserved motifs in this domain. Among these, motifs II and IV are enriched in basic residues and have been implicated in binding with cpSRP43 (35, 45). To test whether these motifs are involved in the interaction with the cpSRP54-cpFtsY complex, we mutated conserved lysine residues in motif II (Lys-367, Lys-377) or motif IV (Lys-444, Lys-445) to aspartate residues. Mutant Alb3-CTD (K367D, K377D) only showed a modestly reduced ability to bind and inhibit the GTPase reaction of the cpSRP54-cpFtsY complex compared with wild type protein (Fig. 5D, blue versus black). In contrast, mutant Alb3-CTD



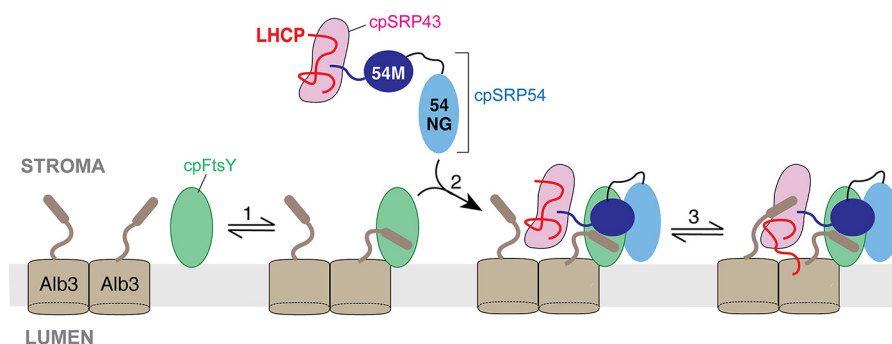
**FIGURE 5. Molecular requirements for the regulatory effect of Alb3-CTD.** A, Alb3-CTD has no effect on the stimulated GTPase reaction from the cpSRP54-NG-cpFtsY complex. Reactions contained 0.5  $\mu\text{M}$  cpSRP54-NG, 3  $\mu\text{M}$  cpFtsY, 0.1 mg/ml PG liposomes, and 100  $\mu\text{M}$  GTP. B, Alb3-CTD has no effect on the GTPase reaction of the cpSRP54-cpFtsY complex in the absence of PG liposomes. Reactions contained 0.1  $\mu\text{M}$  cpSRP54, 3  $\mu\text{M}$  cpFtsY, and 100  $\mu\text{M}$  GTP. C, effect of Alb3-CTD on the stimulated GTPase reaction is not affected by cpSRP43. Reactions used the same conditions as Fig. 4A except for the presence of 5  $\mu\text{M}$  cpSRP43. Error bars indicate S.E. D, effect of wild type and mutant Alb3-CTD on the stimulated GTPase reaction of the cpSRP54-cpFtsY complex. Reactions used 0.1  $\mu\text{M}$  cpSRP54, 0.5  $\mu\text{M}$  cpFtsY, 0.1 mg/ml PG liposomes, and indicated concentrations of wild type or mutant Alb3-CTD. Lines are fits of the data to Equation 4. Error bars indicate S.E.

(K444D, K445D) reduced the GTPase reaction by less than 2-fold (Fig. 5C, red), and mutation of all four lysines in motifs II and IV largely abolished the ability of Alb3-CTD to regulate GTP hydrolysis (Fig. 5D, green). This suggests that motifs II and IV in Alb3-CTD, which has been shown to be important for contacting cpSRP43 (35, 45), are also required for binding and/or regulation of the cpSRP54-cpFtsY complex.

## Discussion

Accurate timing of complex assembly, GTPase activation, and hydrolysis of the SRP and SR GTPases is crucial for efficient protein targeting. Previous studies of the bacterial SRP pathway showed that RNCs bearing SRP-dependent substrate proteins extensively regulate the SRP-SR GTPase cycle, by accelerating SRP-SR complex assembly while also delaying their GTP activation in the complex (38). These cargo-induced allosteric regulations are critical for enhancing the efficiency and fidelity of the co-translational SRP pathway (37, 49). Here, we address whether and how biological cues regulate the GTPase cycles of cpSRP and cpFtsY during the post-translational targeting of LHCPs. Our results strongly suggest that the GTPase cycle of cpSRP54 and cpFtsY is extensively driven by spatial cues from the target membrane and the Alb3 translocase.

We found that anionic phospholipids, specifically PG, induce rapid and stable cpSRP54-cpFtsY complex assembly. On the one hand, this lipid-mediated stimulation of GTPase assembly is analogous to observations in the bacterial SRP pathway,



**FIGURE 6. Proposed model for the recruitment and remodeling of the targeting complex at the membrane translocase site during LHCP targeting and insertion.** Step 1, cpFtsY bound to anionic phospholipids in the thylakoid membrane can associate with Alb3-CTD. For simplicity, cpFtsY molecules that can potentially bind to the second Alb3-CTD in a dimeric Alb3 complex are not depicted. Step 2, membrane-bound cpFtsY binds with high affinity and efficiency to the NG-domain of cpSRP54 in the LHCP-cpSRP complex, bringing the targeting complex to the vicinity of Alb3. Step 3, interaction of Alb3-CTD with the GTPase complex enhances the local effective concentration of cpSRP43, enabling it to also form contacts with the second Alb3-CTD in the Alb3 dimer. Alb3-CTD triggers the selective release of the transmembrane domains of LHCP from cpSRP43 (35), initiating its insertion. Alb3-CTD also delays GTP hydrolysis that would drive cpSRP54-cpFtsY complex disassembly (3), giving the targeting and translocation complexes a prolonged time window for substrate unloading and insertion.

where anionic phospholipids such as PG and cardiolipin also activate FtsY for efficient complex assembly with SRP (41). The lipid-binding motifs that mediate this stimulation, located at the N terminus of the FtsY or cpFtsY NG-domain, are also highly conserved between the bacterial and chloroplast SRP receptors. On the other hand, both lipid flotation and GTPase assays here showed that free cpFtsY binds PG liposomes much more strongly than its bacterial homolog. Previous work also showed that although a large fraction of bacterial FtsY was found in the cytosol in cell fractionation experiments (50), cpFtsY predominantly sediments with thylakoid membrane (42). These observations suggest that cpFtsY associates with its target membrane with much higher affinity than bacterial FtsY. We speculate that a large fraction of the cpFtsY molecules are bound at the thylakoid membrane, where it is pre-organized into an active conformation that can efficiently interact with cpSRP54 and thus ensure rapid delivery of cargo proteins to the thylakoid membrane.

Once cpSRP54 and cpFtsY assemble a stable complex with one another, the targeting complex must be recruited to the Alb3 translocase, which mediates the insertion of LHCPs into the thylakoid membrane. Previously, the observations that cpSRP43 can bind the stromal domain of Alb3 have led to models in which the cpSRP43-Alb3 interaction localizes the targeting complex to the Alb3 translocase (44, 45). However, the low affinity of this interaction ( $K_d$  values of 11–17  $\mu\text{M}$ ) and the predominantly stromal localization of cpSRP43 render such a recruitment mechanism highly inefficient (35, 42, 45). The observation here that Alb3-CTD regulates the cpSRP54-cpFtsY complex at sub-micromolar concentrations indicates that this GTPase complex binds the stromal domain of Alb3 with much higher affinity than cpSRP43. As cpSRP54-cpFtsY complex formation is unaffected by Alb3-CTD, thermodynamic conservation predicts that cpFtsY also binds well to Alb3-CTD, with the same affinity as that of the cpSRP54-cpFtsY complex. Furthermore, the regulatory effect of Alb3-CTD on the GTPases requires PG liposomes and likely occurs when cpFtsY is bound at the thylakoid membrane. Together, these results suggest that membrane-bound cpFtsY is a more likely candidate for recruitment of the targeting complex to the Alb3 translocase. There,

the ability of Alb3-CTD to delay GTPase activation of the cpSRP54-cpFtsY complex could minimize abortive targeting reactions due to premature GTP hydrolysis, allowing a stable cpSRP-cpFtsY complex to accumulate at the site of LHCP unloading and translocation.

As shown in previous work and in the preceding paper (51), the M-domain of cpSRP54 interacts with conserved basic residues in the cpFtsY G-domain; this interaction is required for rapid assembly of a stable cpSRP54-cpFtsY complex that was previously observed only when an artificial stimulant, Nikkol, was used (28). Intriguingly, here we found that both the liposome-induced stimulation of the cpSRP54-cpFtsY complex assembly and the Alb3-mediated regulation of GTP hydrolysis in this complex also require the M-domain of cpSRP54. These results suggest that the phospholipid membrane and the Alb3 translocase selectively recognize the same active conformation of the GTPase complex, in which the cpSRP54 M-domain is properly positioned at the cpFtsY G-domain.

Mutational analysis of conserved basic residues in motifs II and IV of Alb3-CTD showed that these motifs, especially motif IV, are important for the ability of Alb3 to bind and/or regulate the cpSRP-cpFtsY GTPase complex. These same motifs also mediate the interaction of Alb3-CTD with cpSRP43 (35, 44, 45). A potential resolution to these observations is provided by the work of Dunschede *et al.* (44), which showed that full-length Alb3 forms dimers in blue native PAGE. The bacterial homolog of Alb3, YidC, has also been observed to form dimers (52–55). Thus, a plausible model that accommodates the previously identified Alb3-cpSRP43 interaction and the Alb3-GTPase interaction found here is that a dimeric Alb3 complex in the thylakoid membrane can use two CTDs to interact with both cpFtsY and cpSRP43.

Collectively, our results suggest a revised model for the targeting of LHCP mediated by the post-translational cpSRP pathway (Fig. 6). A productive round of targeting likely occurs with cpFtsY molecules that are bound at the thylakoid membrane and can interact with high affinity with Alb3-CTD (Fig. 6, step 1). Phospholipids promote an active conformation of cpFtsY that enables its rapid and stable assembly with cpSRP54, allowing the recruitment and anchoring of the targeting complex at



the translocase site (Fig. 6, *step 2*). This increases the local effective concentration of cpSRP43 and allows it to establish interactions with the other CTD in the dimeric Alb3 complex (Fig. 6, *step 3*). As shown recently, interaction with Alb3-CTD remodels the conformation of cpSRP43 (35); this triggers the selective release of the transmembrane domains of LHCP from cpSRP43 and potentially initiates their insertion, although a loop sequence of LHCP remains bound to cpSRP43 (Fig. 6, *step 3*) (35). Alb3-induced delay in GTP hydrolysis of the cpSRP54-cpFtsY complex prevents the premature disassembly of the targeting complex during this concerted substrate handover and insertion process and thus minimizes abortive targeting reactions.

In contrast to the extensive regulatory effects of phospholipid membrane and the Alb3 translocase, experiments to probe for regulatory effects of cpSRP43 and the cpSRP43-LHCP complex on the cpSRP54/cpFtsY GTPase cycle have largely yielded negative results. The most pronounced effect reported thus far is a 2-fold enhancement of GTPase rate by a truncated mutant of cpSRP43 (42). This is in contrast to the bacterial SRP pathway, in which the cargo extensively regulates complex assembly and GTPase activation between the SRP and SR GTPases. It is attractive to speculate that this difference is related to the greater challenge in achieving fidelity in the co-translational cytosolic SRP pathway. The use of the cargo protein to exert allosteric regulations on the GTPase cycle allows the latter to also impose fidelity checkpoints that discriminate against incorrect cargos and hence improve the specificity of substrate selection by the co-translational SRP (37, 49, 56). In contrast, specific substrate recognition is more easily achieved by the cpSRP43 chaperone in the cpSRP pathway, which allows this pathway to bypass these checkpoints. Continued efforts to explore the similarities and differences between the different SRP systems will allow us to gain a deeper understanding of the mechanism, regulation, and evolution of the novel class of GTPases represented by SRP and the SRP receptor.

## Experimental Procedures

**Protein Expression and Purification**—Wild type cpSRP54 and cpFtsY from *Arabidopsis thaliana* were overexpressed and purified as described previously (29). N-terminal deletion mutants of cpFtsY and cpSRP54-NG were constructed using the QuikChange mutagenesis protocol following the manufacturer's instructions (Agilent Technologies) and were expressed and purified as for wild type proteins. Residues 339–462 of Alb3 (Alb3-CTD) were cloned into pET28b (Novagen) between NcoI and XhoI restriction sites with a C-terminal His<sub>6</sub> tag. Alb3-CTD was expressed in BL21-DE3\* cells (Invitrogen) at 37 °C, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (Bio-pioneer) at an A<sub>600 nm</sub> of 0.6. The cells were harvested after growing for an additional 3–4 h and resuspended in buffer containing 50 mM KHEPES (pH 7.5), 500 mM NaCl, 5 mM MgCl<sub>2</sub>, and 5% glycerol. Cells were lysed by French press (Thermo Scientific). Clarified lysate was bound to 2 ml of nickel-nitrilotriacetic acid resin/liter cells (Qiagen), washed with Ni-buffer (50 mM KHEPES (pH 7.5), 1 M NaCl, 20 mM imidazole, and 5% glycerol), and eluted with Ni-buffer containing 200 mM imidazole. Pooled peak elution fractions were dialyzed in

50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM DTT, and 5% glycerol and purified over a cation exchange Mono S column (GE Healthcare) using a linear gradient of 150–600 mM NaCl over 20 column volumes. Alb3-CTD eluted at ~300 mM NaCl.

Plant leaf PG (Lipid Products) liposomes were prepared by six freeze-thawing cycles of the lipid suspension in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, followed by extrusion over an 100-μm filter 21 times. Liposomes were prepared at a stock concentration of 10 mg/ml (40, 41).

**GTPase Assays**—All GTP hydrolysis reactions were performed in assay buffer (50 mM KHEPES (pH 7.5), 150 mM KOAc, 2 mM Mg(OAc)<sub>2</sub>, 10% glycerol, 2 mM DTT) at 25 °C as described (36). Stimulated GTP hydrolysis reactions were carried out using limiting wild type or mutant cpSRP54 (0.1 μM), indicated cpFtsY concentrations, and 100 μM GTP doped with [γ-<sup>32</sup>P]GTP. The dependence of observed rate constants (*k*<sub>obsd</sub>) on cpFtsY concentration were fit to Equation 1,

$$k_{\text{obsd}} = k_{\text{cat}} \times \frac{[\text{cpFtsY}]}{K_m + [\text{cpFtsY}]} \quad (\text{Eq. 1})$$

in which *k*<sub>cat</sub> is the rate constant at saturating cpFtsY concentrations, and *K*<sub>m</sub> is the concentration of cpFtsY required to reach the half-maximal rate.

Effects of PG on the stimulated GTPase reaction were measured at sub-saturating concentrations of cpSRP54 (0.1 μM) and cpFtsY (≤0.3 μM), at which the observed reaction is rate-limited by complex assembly. The PG concentration dependence of the observed rate constants (*k*<sub>obsd</sub>) were fit to Equation 2,

$$k_{\text{obsd}} = k_{\text{max}} \times \frac{[\text{PG}]}{K_{1/2} + [\text{PG}]} \quad (\text{Eq. 2})$$

in which *k*<sub>max</sub> is the maximal rate constant at saturating PG concentration, and *K*<sub>1/2</sub> is the PG concentration required to reach the half-maximal rate.

Basal GTPase reactions of free cpSRP54 or cpFtsY were measured under single turnover conditions with trace [γ-<sup>32</sup>P]GTP and protein in excess over GTP, as described previously (36). The protein concentration dependence of observed rate constants (*k*<sub>obsd</sub>) were fit to Equation 3,

$$k_{\text{obsd}} = k_{\text{max}} \times \frac{[\text{protein}]}{K_{1/2} + [\text{protein}]} \quad (\text{Eq. 3})$$

in which *k*<sub>max</sub> is the maximal rate constant at saturating protein concentrations, and *K*<sub>1/2</sub> is the protein concentration required to reach the half-maximal rate.

The Alb3-CTD concentration dependence of the observed GTPase rate constant (*k*<sub>cat, obsd</sub>) was fit to Equation 4,

$$k_{\text{cat, obsd}} = k_1 + (k_2 - k_1) \times \frac{([G] + [A] + K_i) - \sqrt{([G] + [A] + K_i)^2 - 4[A][G]}}{2[G]} \quad (\text{Eq. 4})$$

in which *k*<sub>1</sub> is the GTPase rate constant in the absence of Alb3-CTD; *k*<sub>2</sub> is the GTPase rate constant with Alb3-CTD bound to the GTPase complex, [G] and [A] denote the initial concentra-

tions of the cpSRP54·cpFtsY complex and Alb3-CTD, respectively, and  $K_i$  is the inhibition constant for Alb3-CTD.

**Fluorescence Measurements**—Single cysteine mutants of cpSRP54 and cpFtsY were labeled and purified as described previously (29). Labeling efficiency was typically >80% for all dyes, with <10% background. All fluorescence measurements were performed at 25 °C in assay buffer (50 mM KHEPES (pH 7.5), 150 mM KOAc, 2 mM Mg(OAc)<sub>2</sub>, 10% glycerol) on a FluoroLog 3-22 spectrofluorometer (Jobin Yvon). To form the GTP-bound cpSRP54·cpFtsY complex, 2 mM GTP (Sigma) was used; GDP released during the course of the reaction was minimal, as explained previously (29). FRET and fluorescence measurements based on DACM-labeled cpFtsY used an excitation wavelength of 380 nm and an emission wavelength of 450 nm. Fluorescence measurements based on acrylodan-labeled cpSRP54 were measured using an excitation wavelength of 370 nm and an emission wavelength of 450 nm.

Equilibrium titrations for cpSRP54·cpFtsY complex formation in the presence of plant PG liposomes were carried out using fluorescence enhancement of DACM labeled at cpFtsY Cys-321 upon binding unlabeled cpSRP54. The cpSRP54 concentration dependence of observed fluorescence change ( $\Delta F_{\text{obsd}}$ ) was fit to Equation 5,

$$\Delta F_{\text{obsd}} = \Delta F \times \frac{([Y] + [54] + K_d) - \sqrt{([54] + [Y] + K_d)^2 - 4[Y][54]}}{2[Y]} \quad (\text{Eq. 5})$$

in which Y is cpFtsY; 54 is cpSRP54;  $\Delta F$  is the fluorescence change at saturating cpSRP54 concentrations; and  $K_d$  is the equilibrium dissociation constant of the complex.

Titration in the absence of PG were carried out using FRET between DACM labeled at cpFtsY Cys-321 and BODIPY labeled at cpSRP54 Cys-234. The FRET efficiency ( $E$ ) was calculated as described previously (29, 57). The cpSRP54 concentration dependence of observed FRET ( $E_{\text{obsd}}$ ) was fit to Equation 6,

$$E_{\text{obsd}} = E_1 \times \frac{[\text{cpSRP54}]}{K_d + [\text{cpSRP54}]} \quad (\text{Eq. 6})$$

in which  $E_1$  is the FRET value with saturating protein and  $K_d$  is the equilibrium dissociation constant of the complex.

Equilibrium titrations for formation of the cpSRP54·cpFtsY complex in the absence and presence of Alb3-CTD were carried out using fluorescence enhancement of acrylodan labeled at cpSRP54 Cys-234 upon binding cpFtsY. The cpFtsY concentration dependence of observed fluorescence change ( $\Delta F_{\text{obsd}}$ ) was fit to Equation 5, except that the term [cpFtsY] in the denominator is replaced by [cpSRP54].

**Lipid Flotation Assays**—Flotation assays were performed as described previously (41). Briefly, cpSRP54 and cpFtsY at 20  $\mu\text{M}$  were incubated for 2 h at room temperature in the presence of 1 mM GppNHp to enable complex formation. Freshly extruded liposomes were added to proteins or pre-formed complexes and further incubated at 37 °C for 30 min. The sample was then loaded on a 20–40% OptiPrep™ (Sigma) gradient and ultracentrifuged at 42,000 rpm in a Beckman SWTI 55 rotor for 3 h.

Immediately after centrifugation, 600- $\mu\text{L}$  fractions were collected, precipitated with 15% trichloroacetic acid (Sigma), and analyzed by SDS-PAGE. The data were quantified using the ImageJ program (58).

**Author Contributions**—S. S. conceived and coordinated the study and wrote the paper. S. C. designed, performed, and analyzed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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